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Award Number: DAMD17-99-1-9143

TITLE: Role of Melatonin in the Prevention of Breast Cancer in
Patients with Cystic Breast Disease

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REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20040706 014

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 074-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)			2. REPORT DATE September 2003		3. REPORT TYPE AND DATES COVERED Annual (1 Sep 2002 - 31 Aug 2003)	
4. TITLE AND SUBTITLE Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease			5. FUNDING NUMBERS DAMD17-99-1-9143			
6. AUTHOR(S) Robert L. Wells, Ph.D.						
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Colorado State University Fort Collins, Colorado 80523 E-Mail: robwells@colostate.edu			8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER			
11. SUPPLEMENTARY NOTES						
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The primary objectives of this study are to establish a clinical breast cyst fluid (BCF) sample bank among patients with gross cystic breast disease (GCBD) in order to study the relationship between GCBD, melatonin and related growth agents in BCF, and breast cancer risk. BCF samples from participating patients will be used to elucidate the contribution of melatonin and related growth agents (EGF, TGF-beta, DHEA-S) to the oncostatic effects of BCF on MCF-7 human breast cancer cells. This project is currently in its final year, which was added as a no-cost extension to the original study. Progress through year 3 included continued patient enrollment and completion of biochemical assays and growth experiments for incoming BCF samples. Patient recruitment is scheduled for completion in the near future and statistical data analyses will proceed in this final grant year. Preliminary results obtained from interim statistical analyses are consistent with previously reported findings. In summary, cell culture growth experiments using BCF samples and corresponding biochemical composition assays continued in year 3. This project is scheduled for completion in the upcoming grant year.						
14. SUBJECT TERMS Breast Cancer, Melatonin, Gross Cystic Breast Disease, MCF-7 Cells					15. NUMBER OF PAGES 13	
					16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Cover	1
SF 298	2
Table of Contents	3
Introduction	4
Body	4
Key Research Accomplishments	7
Reportable Outcomes	7
Conclusions	7
Literature Cited	9
Table 1	11
Table 2	12
Table 3	12
Appendix	13

INTRODUCTION

Long term exposure of breast tissue to estrogen plays a major role in breast tumor formation, although risk factors linked with chronic estrogen exposure only account for a portion of disease occurrence. Other hormones and growth factors are likely to be related to unidentified breast cancer risk factors. The pineal hormone, melatonin, exerts antiproliferative and anticarcinogenic effects via several proposed mechanisms, including the modified secretion of growth factors that are critical for breast tumor development. Prior research suggests that women with high endogenous melatonin levels may have lower breast cancer risks and that melatonin may be an effective therapeutic agent for breast cancer treatment. Women with palpable breast cysts have a 2-6 fold increased risk of developing breast cancer. Despite the identification of several growth agents in breast cyst fluid (BCF) that appear to segregate based on electrolyte (Na,K) composition, it is still unclear what role BCF plays in breast tumor development. BCF exerts an inhibitory effect on the proliferation of breast cancer cells *in vitro*. We hypothesize that this effect is due to the presence of melatonin in BCF, which stimulates production of transforming growth factor-beta (TGF-beta) in human breast cancer cells. The two major goals of this study encompass clinical and laboratory-based activities. The clinical effort will establish a cyst fluid sample bank to evaluate the relationship between BCF composition and breast cancer risks. The laboratory effort will use the BCF samples to elucidate the contribution of melatonin to the oncostatic effects of cyst fluids in a breast cancer cell model (MCF-7). The relationship between melatonin and specific growth factors in BCF will also be assessed. The specific aims are to: 1) create a BCF sample bank among patients with gross cystic breast disease in order to perform a prospective study of the relationship between gross cystic breast disease, melatonin and related growth agents in BCF, and breast cancer risk; 2) determine melatonin, TGF-beta, epidermal growth factor (EGF), dehydroepiandrosterone-sulfate (DHEA-S), and electrolyte (Na,K) concentrations in BCF samples; 3) perform cell proliferation studies in MCF-7 breast cancer cells treated with BCF and compare growth rates with melatonin and other analytes in BCF; 4) determine whether TGF-beta synthesis is enhanced in BCF-treated MCF-7 cells and whether the enhancement is related to melatonin levels in the BCF; 5) determine the relationship between melatonin and growth agents (EGF, TGF-beta, DHEA-S) in BCF; 6) quantify Na and K concentrations in BCF to determine whether low breast cancer risk BCF (high Na:K ratio) contains elevated levels of melatonin and TGF-beta, and reduced concentrations of EGF and DHEA-S; and 7) determine whether melatonin in BCF is associated with known risk factors for breast cancer in patients with gross cystic breast disease.

BODY

The statement of work for this project includes four tasks. Each of these tasks has been initiated and work is on-going according to the study design. This project is currently in an extension year so that patient recruitment goals and other objectives can be met. The major objectives of each task and subtask are outlined below. A summary of progress in year 3 is summarized following each task description.

Task 1: Establish a sample bank for breast cyst fluids of patients with gross cystic breast disease (Specific Aim 1).

Sub-Task A. Obtain final approval from institutional review boards for modifying existing human informed consent procedures.

Sub-Task B. Establish protocols for breast cyst fluid (BCF) sample collection, handling, storage, chain of custody, shipment and record keeping.

Sub-Task C. Initiate patient recruitment.

Sub-Task D. Process BCF samples.

A sample bank for breast cyst fluids from patients with gross cystic breast disease was established in grant Year 1 and patient recruitment remains on-going. Recruitment was initiated in Year 1 at the University of Washington Medical School (UW). Approval for recruitment at two additional clinics, the University of Colorado Health Sciences Center (UCHSC) in Denver, Colorado (Tina Finlayson, MD, On-site Coordinator) and the Breast Diagnostic Center at Poudre Valley Hospital (PVH) in Fort Collins, Colorado (Winfield Craven, MD, On-site Coordinator), was initiated in Year 3. We anticipate that patient recruitment will be completed in the near future and Task 1 will be complete. At that point, work on the remaining tasks will be finalized.

Task 2. Determine melatonin, TGF-beta 1 and 2, EGF, DHEA-S, and electrolyte (Na,K) concentrations in breast cyst fluid samples (Specific Aim 2).

Sub-Task A. Standardize assay procedures.

Sub-Task B. Perform biochemical assays of melatonin and growth-related agents.

Sub-Task C. Determine ionic composition (Na,K) of BCF samples by AA spectroscopy.

Task 2 focuses on the determination of melatonin, electrolytes (Na,K), and other growth related agents (EGF, TGF-beta 1 and 2, and DHEA-S) in BCF samples. The assay protocols for each of the analytes have been established and were reported previously. Biochemical assays for BCF constituents will remain on-going until patient recruitment and sample acquisition has been completed. Work on this task is proceeding as specified in the statement of work.

Task 3. Perform cell proliferation studies in MCF-7 breast tumor cells treated with breast cyst fluids to compare cell growth effects with melatonin and growth factors in BCF (Specific Aims 3 and 4).

Sub-Task A. Determine initial dose-range toxicity and proliferation response relationships in MCF-7 cells treated with BCF.

Sub-Task B. Perform cell proliferation and TGF-beta response studies with BCF samples.

Sub-Task C. Calculate growth indices for each BCF sample.

Task 3 focuses on the performance of experiments *in vitro* to determine the growth properties of BCF samples in human breast cancer (MCF-7) cells. The experimental protocols for this task were established in previous grant years. Assessment of the growth regulatory properties of BCF samples was on-going in Year 3 and will continue until patient recruitment is complete. Work on Task 3 is proceeding as specified in the statement of work.

Task 4. Perform biostatistical data analyses to: determine the relationship between melatonin and growth-related substances in BCF (Specific Aim 5); determine whether low breast cancer risk (high Na:K ratio) BCFs contain higher levels of melatonin and TGF-beta and lower levels of EGF and DHEA-S (Specific Aim 6); and determine whether melatonin

in BCF is associated with known risk factors for breast cancer in patients with gross cystic breast disease (Specific Aim 7).

Sub-Task A. Develop a computerized database; enter cell proliferation, biochemical, clinical, and questionnaire data into database and validate data.

Sub-Task B. Calculate descriptive statistics for melatonin, TGF-beta, EGF, and DHEA-S concentrations in BCF samples.

Sub-Task C. Compare mean cell counts in replicate control and treatment groups via t-tests; calculate a correlation matrix for each combination of growth index, melatonin, TGF-beta, EGF, and DHEA-S data; obtain the best fit of each combination to a numerical equation using linear regression.

Sub-Task D. Perform multivariate linear regression analyses.

Sub-Task E. Classify patients into low and high risk (Type 1 and 2) breast cyst groups using Na:K ratios; perform t-tests on mean melatonin concentrations in Type 1 and 2 BCF; perform similar analyses for growth index, TGF-beta, DHEA-S, EGF data.

Sub-Task F. Stratify patients into Type 1 and 2 groups; perform linear regression of growth index or melatonin data vs. EGF, TGF-beta, or DHEA-S in each group; compare regression coefficients.

Sub-Task G. Stratify patients into levels of each breast cancer risk factor and compare mean melatonin concentrations among each level via t-test, ANOVA, or multiple linear regression analyses.

Task 4 focuses on statistical data analyses. Work initiated in previous grant years includes the development of computerized data bases that compile BCF biochemical assay and cell proliferation data, as well as questionnaire responses. Because data accrual is still ongoing, it would have been impractical to conduct extensive biostatistical data analyses at this time. However, some activities in support of Task 4 were performed in year 3. Descriptive statistics for a subset of BCF samples are presented in Table 1. The mean volume (\pm standard deviation) of BCF samples collected approximately mid-way through Year 3 (n=118) was approximately 7.0 ± 7.4 mls. In a small proportion (19%) of the BCF samples, the ratio of sodium to potassium (Na:K) was greater than than 3 (Table 2). Cysts with Na:K ratios below 3 have been associated with elevated breast cancer risks in some previous studies (Table 3) (Angeli et al., 1994; Bruzzi et al., 1997; Caraci et al., 1994; Dixon et al., 1985; Fleisher et al., 1984; Naldoni et al., 1990). Our preliminary results indicate that cysts with a Na:K ratio less than 3 have elevated concentrations of EGF and DHEA-S compared with BCF samples with Na:K ratios greater than 3 (Tables 2 and 3). These results are consistent with one of our original study hypotheses and with previous studies of BCF composition (Angeli et al., 1992; Belanger et al., 1990; Boccardo et al., 1988; Bradlow et al., 1979; Lai et al., 1989; Lai et al., 1990; Miller et al., 1983; Zanardi et al., 1996). These preliminary findings are reassuring in that they provide this study with some degree of external validity although no conclusions can be made until recruitment is completed and our the entire data set is analyzed statistically. This task will be accomplished within the upcoming grant year.

KEY RESEARCH ACCOMPLISHMENTS

- Patient enrollment continued in year 3 and is near completion; two new recruitment sites were added;
- Biochemical assays for each analyte have been performed on newly acquired BCF samples;
- BCF samples from recruited patients were used to treat MCF-7 cells in culture;
- Data were compiled in a computerized data base that includes: biochemical constituents in BCF samples, growth experiments, and breast cancer risk factor data obtained via questionnaire. Some interim data analyses were performed and preliminary results are consistent with several previous studies.

REPORTABLE OUTCOMES

- The human breast cyst fluid sample bank, established in year 1, was expanded through year 3. This sample repository serves as a biological resource for performing studies on the relationship between breast cyst fluid composition and breast cancer risk in women.
- The study authors presented a poster at the Era of Hope Department of Defense Breast Cancer Research Program Meeting in Orlando, Florida in September, 2002.
- An abstract describing this study was published as part of the Proceedings of the Era of Hope Department of Defense Breast Cancer Research Program (Burch et al, 2002) (see Appendix).

CONCLUSIONS

Breast cancer remains a leading cause of cancer mortality among women worldwide. Because the known risk factors for breast cancer only account for a portion of the disease occurrence, research is needed to determine the role of hormones and related growth agents other than estrogen that are likely to be associated with as yet unidentified breast cancer risk factors. Women with palpable breast cysts have a 2-6 fold increased risk of developing breast cancer, although studies indicate that breast cyst fluid has antiproliferative properties. Further research is needed to evaluate the role of cyst fluid composition in breast tumor development.

Our long term goal is to study the relationship between cyst fluid composition and breast cancer risk. The primary objectives of this study are to establish a clinical breast cyst fluid sample bank among patients with gross cystic breast disease, and determine the relationship between the biochemical composition of BCF and their growth properties. BCF samples from this repository will continue to be used to elucidate the contribution of melatonin and related growth agents to the oncostatic effects of cyst fluids in a human breast cancer cell model. Each task described in the statement of work has been initiated and specific subtasks have either been completed or remain on-going in accordance with the study design. Preliminary results obtained from interim statistical analyses are consistent with previously reported findings. Patient recruitment is scheduled for completion in the near future and statistical data analyses will proceed in the upcoming grant year. This study will add to our understanding of breast cancer biology. The results

will be valuable for evaluating the potential predictive and therapeutic role of BCF constituents, including melatonin, in breast cancer risk.

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Table 1. Biochemical Constituents in Breast Cyst Fluid Samples

Variable	N	Range	Mean	Standard Deviation	Standard Error of the Mean
Volume (mls)	118	0.5 – 41.0	7.0	7.4	0.7
Na:K Ratio	98	0.1 – 15.0	1.7	3.0	0.3
Melatonin	96	13.2 – 615.0	156.3	129.0	13.2
EGF (ng/ml)	70	0.2 – 529.2	191.8	110.0	13.1
DHEAS (ug/ml)	104	0.6 – 581.4	73.5	93.3	9.2
TGF-B1 (pg/ml)	96	19 – 5,935	1,033	1,311	134
TGF-B2 (pg/ml)	103	11 – 106,352	10,016	15,196	1,497

TGF-B: Transforming Growth Factor-Beta EGF: Epidermal Growth Factor
DHEA-S: Dehydroepiandrosterone-Sulfate

**Table 2. Biochemical Constituents in Breast Cyst Fluid Samples
(Only Samples with Na:K Ratios ≥ 3.0)**

Variable	N	Range	Mean	Standard Deviation	Standard Error of the Mean
Volume	19	2.0 – 41.0	7.3	10.1	2.3
Na:K Ratio	19	3.0 – 15.0	6.6	3.8	0.9
Melatonin	18	17.7 – 427.7	105.3	107.3	25.3
EGF (ng/ml)	10	0.2 – 188.4	90.6	54.8	17.3
DHEAS (ug/ml)	19	0.6 – 36.6	13.1	10.5	2.4
TGFB1 (pg/ml)	18	137 – 5,472	1,681	1,629	384
TGFB2 (pg/ml)	18	2,221 – 32,104	12,433	9,973	2,351

TGF-B: Transforming Growth Factor-Beta EGF: Epidermal Growth Factor
DHEA-S: Dehydroepiandrosterone-Sulfate

**Table 3. Biochemical Constituents in Breast Cyst Fluid Samples
(Only Samples with Na:K Ratios < 3.0)**

Variable	N	Range	Mean	Standard Deviation	Standard Error of the Mean
Volume	99	0.5 – 30.5	6.9	6.9	0.7
Na:K Ratio	79	0.1 – 2.9	0.5	0.6	0.1
Melatonin	78	13.2 – 615.0	168.0	131.3	14.9
EGF (ng/ml)	60	7.5 – 529.2	208.7	107.6	13.9
DHEAS (ug/ml)	85	1.0 – 581.4	87.0	98.2	10.7
TGFB1 (pg/ml)	78	19 - 5,343	884	1,188	135
TGFB2 (pg/ml)	85	11 - 106,352	9,504	16,086	1,745

TGF-B: Transforming Growth Factor-Beta EGF: Epidermal Growth Factor
DHEA-S: Dehydroepiandrosterone-Sulfate

APPENDIX

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

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Benign breast disease affects one of every two women and those who develop palpable breast cysts (gross cystic breast disease) have a 2-4 fold increased risk of developing breast cancer. Breast cyst fluids (BCFs) contain hormones, growth factors, and other agents linked with increased breast cancer risks. Our research was the first to identify melatonin in BCF and we hypothesize that it is responsible for BCF's previously observed antiproliferative properties *in vitro*. The objectives of this study are to: 1) establish a clinical BCF sample bank in order to study of the relationship between gross cystic breast disease, melatonin and related growth agents in BCF, and breast cancer risk; 2) determine melatonin, transforming growth factor(TGF)-beta 1&2, epidermal growth factor(EGF), and dehydroepiandrosterone-sulfate (DHEA-S) concentrations in BCF samples; 3) determine the relationships among melatonin and these growth agents (EGF, TGF-beta, DHEA-S) in BCF; 4) perform cell proliferation studies in MCF-7 human breast cancer cells treated with BCF and compare growth rates with melatonin and other BCF constituent concentrations; 5) determine whether TGF-beta synthesis is altered in BCF-treated MCF-7 cells and whether changes are related to melatonin or other agents the BCF; 6) quantify sodium (Na) and potassium (K) concentrations in BCF to determine whether BCF with a high Na:K ratio (associated with low breast cancer risk in some studies) contains elevated levels of melatonin and TGF-beta, and reduced concentrations of EGF and DHEA-S; and 7) determine whether known breast cancer risk factors are more common among patients with low BCF melatonin levels. Assay procedures have been established for melatonin, TGF-beta 1&2, EGF, DHEA-S, and electrolytes in BCF. Time course, dose-response, and repeatability experiments have been completed and a bioassay protocol has been established for treatment of MCF-7 breast cancer cells with BCF samples. Patient enrollment was initiated in year 1 and remains on-going. Known risk factors only explain about forty percent of breast cancer incidence. Hormones other than estrogen and related growth factors may be linked with unidentified breast cancer risks. Results from this study will help elucidate the inhibitory role of BCF and melatonin on human breast cancer cell growth. The analysis of BCF constituents provides an opportunity to evaluate predictive markers of breast cancer risk and identify potential targets of therapeutic intervention.

Supported by the U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9143.